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Colonization of torrefied grass fibers by plant-beneficial microorganisms

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ABSTRACT

This study aimed to assess the colonization of thermally treated (i.e. torrefied) grass fibers (TGFs), a new prospective ingredient of potting soil. Eleven bacterial strains and one fungus, *Coniochaeta ligniaria* F/TGF15, all isolated from TGF or its extract after inoculation with a soil microbial community, were tested for their ability to colonize TGF. Surprisingly, none of these bacteria were able to directly colonize TGF either as single inoculants or as a consortium. Furthermore, bacterial persistence or growth in TGF was not improved by the addition of nutrients or a surfactant. Only extensive washing of the substrate, presumably removing bacteriostatic or bactericidal compounds, allowed bacterial growth on the fibers. Strikingly, the fungal strain consistently colonized TGF up to high densities (up to 10^{10} CFU per g dry TGF). Given the unique capacity of this fungus to degrade toxic compounds including phenols, TGF was colonized with it for different periods of time, after which a consortium of seven selected bacterial isolates was added. Co-presence of the fungus, or 3 and 24 h pre-colonization with it, was insufficient to create a habitable environment for the bacterial consortium. However, fungal pre-colonization of minimally 3 days allowed the bacterial consortium to colonize the TGF at numbers up to 10^9 to 10^{10} CFU per g dry substrate. The resultant bacterial community consisted of at least four strains, i.e. *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, and *Methylobacterium radiotolerans* 56/TGF10, as shown by PCR of colonies on plates and PCR–DGGE profiling. Two persisters, *S. plymuthica* 23/TGE5 and *P. corrugata* 31/TGE5, were highly antagonistic towards several phytopathogenic fungi. Thus, a microbial community with plant-beneficial potential was established on TGF, provided that the fungus *C. ligniaria* F/TGF15 first creates habitable space in the matrix.

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1. Introduction

Torrefied grass fibers (TGFs) have been suggested as a new substrate for plant growth, which might replace peat in potting soil (Trifonova et al., 2008a). Grass is grown worldwide and it represents an easily renewable substrate, in contrast to peat which requires a long-term restoration. By exposing dried grass fibers to a heat treatment, i.e. torrefaction, for 1 h at 240 °C, the fibers are transformed into a stable matrix which is fairly protected against decay. Torrefied grass fibers have a high water holding capacity, which is comparable to that of peat. However, a disadvantage is the occurrence of phytotoxic compounds after the heat treatment, which on the other hand may be decreased by microbial activity (Trifonova et al., 2008a,b).

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The conditions of sterility of the TGF just after the heat treatment offer an ideal situation for the introduction of selected microorganisms with desired properties. Thus, survival of introduced microorganisms will not be hampered by competition with an autochthonous microbial population. A crucial question which arose is: how should an ideal microbial community be composed, i.e. what are the requirements concerning the community composition leading to a stable community, and, what are the desired properties of the individual microorganisms that make up the consortium? A first requirement might be the microbial ability to break down the phytotoxic compounds that may be present on the TGF. Finally, microorganisms that are equipped with plant-beneficial properties would give the substrate an added value. Plant-growth-promoting bacteria, as well as plant pathogen inhibiting microorganisms are prime candidates to make part of the microbial consortium.

A stable microbial community in TGF should, theoretically at least, consist of a consortium comprising both *r* and *K* strategists. Novel niches provided by the TGF system will first be colonized by a limited number of species with high growth rates and high fecundity (*r* strategists). These organisms are highly opportunistic, showing little specialization. They are expected to rapidly utilize the easily available substrates (Gadgil and Solbrig, 1972). Early stages of colonization of novel habitats are thus often characterized by low diversity and evenness values (Van Elsas et al., 2007). With time, an increasing number of pre-existing or newly formed niches will be filled, and increasing demands will be placed on the competitive abilities of individual populations since easily available substrates become depleted. As a result, the diversity and evenness levels in the system will increase. In the later stages of colonization, *K* strategists, defined by their lower growth rates and fecundity, may establish in the community (Gadgil and Solbrig, 1972). Thus, it is hypothesized that stable and mature microbial communities should comprise microorganisms of different ecophysiological types, including *r*-strategists as well as *K*-strategists.

The most abundant easily available carbon sources present in the TGF are acetic acid and formic acid (Trifonova et al., 2008a). These compounds can theoretically serve as nutrient and energy sources for many microorganisms. Moreover, phytotoxic compounds, formed as a side effect of the heating process, are present. Hence, microorganisms that utilize these compounds are ideal candidates to be the first colonizers of the substrate.

In this study, we aimed to colonize TGF with a microbial consortium assembled on the basis of assumptions about ecological behaviour and function. Thus, we aimed for a consortium that is able to:

(1) successfully colonize TGF and persist on it,
(2) reduce the phytotoxicity of the substrate,
(3) have plant-beneficial properties.

Thus, 11 bacterial and 1 fungal strain were selected among – in total – 88 strains that originated from enrichment cultures in TGF and its extract (Trifonova et al., 2008a,b). The criteria used to select these strains were (1) the capacity to improve lettuce seed germination in TGF extract, (2) the capability to grow on and transform several phytotoxic compounds present in TGF, and (3) growth on acetic acid and/or formic acid as the sole carbon source(s). In addition, we took into consideration these being either *r* or *K* strategists as well as their capacity to inhibit phytopathogenic fungi.

2. Material and methods

2.1. *Bacterial isolates*

Eleven bacterial strains were obtained from sequential enrichment cultures (Trifonova et al., 2008a). They belonged to the α-proteobacteria (*Rhizobium radiobacter* 43/TGE20 and *Methylobacterium radiotolerans* 56/TGF10), γ-proteobacteria (*Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, and *Stenotrophomonas maltophilia* 34/TGE5), the actinobacteria (*Leifsonia xyli* subsp. *xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15, *Agrococcus casei* 72/TGF15, and *Agromyces aurantiacus* 95/TGF15), and the *Flavobacterium–Cytophaga* group (*Flavobacterium denitrificans* 48/TGE20). They were all able to enhance lettuce seed germination (Trifonova et al., 2008b), but they differed in several characteristics described in Table 1.

Each strain was grown on R2A (Difco, Detroit, MI, USA). Short-term storage was at 4 °C and long-term storage in 20% glycerol at −80 °C.

2.2. *The fungus Coniochaeta ligniaria F/TGF15*

C. ligniaria F/TGF15 was isolated by sequential enrichment on TGF and identified by its 18S ribosomal RNA gene sequence (Trifonova et al., 2008b). *C. ligniaria* is an ascomycete (former genus name is *Phialophora*), which degrades lignocelluloses and is often found on woody substrates. It produces cream-coloured to orange colonies with purplish brown tinges in the centre (Domsch et al., 2007; Jensen, 1985). *C. ligniaria* is a slow-growing organism. This isolate gave significant enhancement of lettuce seed germination and can utilize many of the phytotoxic compounds present in TGF (Trifonova et al., 2008b) (see Table 1). The fungus was maintained on PDA medium (Potato Dextrose Agar 39 g/l, Oxoid, London, UK) at 20 °C in the dark.

2.3. *In vitro antagonism of selected TGF bacteria towards a range of fungi*

Antagonistic activity of selected bacterial strains towards several fungi was determined in an *in vitro* assay on PDA. Three fungi isolated from TGF, namely *Fusarium* sp., *Penicillium* sp. and *Rhizopus* sp., six phytopathogenic fungi (namely two strains of *Fusarium oxysporum* sp. *radicis lycopersici*, *Pythium ultimum*, *Pythium aphanidermatum*, *Rhizoctonia solani*, and *Verticillium dahliae*), and one antagonistic fungus, namely *Trichoderma harzianum*, were assessed in this test. They were grown on PDA medium for a period of 2–10 days at 20 °C. A suspension of hyphal fragments and/or spores from each fungal strain was obtained by adding 10 ml of sterile distilled water to the Petri dish. After scraping with a spatula, the suspension was diluted 10-fold in 0.85% NaCl. Aliquots (500 μl) of diluted suspension were added to Petri dishes and mixed

Table 1 – Characteristics of isolates.

Isolate no.	Name of closest affiliate	Genbank accession no.	Origin	Number of successful germination assays/total[a]	r or K strategist[b]	Taxonomic group	Number of toxic compounds used[c]	Growth on A, F, A + F[d]
15/TGE5	*Pseudomonas putida*	EU293384	TGE5	4/4	r	Gram −	3	A + F
23/TGE5	*Serratia plymutica*	EU293380	TGE5	3/4	r	Gram −	4	A, A + F
31/TGE5	*Pseudomonas corrugata*	EU293383	TGE5	3/5	r	Gram −	2	F, A + F
34/TGE5	*Stenotrophomonas maltophilia*	EU293366	TGE5	2/2	r	Gram −	0	A + F
43/TGE20	*Rhizobium radiobacter*	EU293379	TGE20	2/2	r	Gram −	2	A, F, A + F
48/TGE20	*Flavobacterium denitrificans*	EU293378	TGE20	2/2	r	Gram −	0	A + F
56/TGF10	*Methylobacterium radiotolerans*	EU293389	TGF10	3/3	K	Gram −	2	F, A + F
66/TGF15	*Leifsonia xyli subsp. xyli*	EU293373	TGF15	2/2	K	Gram +	6	A + F
70/TGF15	*Mycobacterium anthracenicum*	EU293382	TGF15	2/2	K	Gram +	0	A, F, A + F
72/TGF15	*Agrococcus casei*	EU293376	TGF15	3/3	K	Gram +	1	A + F
95/TGF15	*Agromyces aurantiacus*	EU293381	TGF15	4/4	K	Gram +	3	A + F
F/TGF15	*Coniochaeta ligniaria*	EU450836	TGF15	2/2	K	Fungus	6	A, A + F

[a] Lettuce seed germination assay with significant improvement of germination ($P = 0.05$)/total number of assays performed (Trifonova et al., 2008b).

[b] Assumption based on general information about the species.

[c] Toxic compounds: phenol, 2-methoxyphenol, 2,6-dimethoxyphenol, 2-furalaldehyde, pyrrole-2-carboxaldehyde, and furan-2-methanol (Trifonova et al., 2008b).

[d] Growth in mineral salt medium with acetic acid (A) (1%), formic acid (F) (1%), and acetic acid (0.5%) + formic acid (0.5%) (A + F) as sole carbon source (Trifonova et al., 2008a).

with 10 ml of PDA just before solidifying. The bacterial strains were inoculated at the edges of the inoculated Petri dishes. Each combination was performed in two replicates. After 5 days of incubation at 20 °C, inhibition zones around the inoculated bacteria were measured.

2.4. *Preparation of TGF for colonization experiments*

Grass was collected in August 2003 from unfertilized grassland on a peaty clay soil near Wageningen, The Netherlands (Trifonova et al., 2008a). The biomass was harvested as a first cut and consisted of a mixture of species (mainly grasses, rushes, sedges, and herbs) with a dry matter content of 37.5%. The harvested material was dried at 105 °C, ground in a hammermill to pieces <1 mm and stored until use for torrefaction experiments.

Samples were torrefied in a preheated Carbolite muffle furnace (Model CWF 1100) at 240 °C during 1 h. The pH of the thus prepared TGF was 5.4.

For all colonization experiments described below, TGF samples of 0.5 g were placed in 50-ml vessels and sterilized by gamma-irradiation (25 kGy). Subsequently, 1.6 ml of inoculant suspension prepared as described below was added, thus establishing 60% of the substrate's water holding capacity. The system thus established was homogenized with a sterile needle and incubated at 25 °C in the dark. Treatments in all experiments were done in two replicates.

2.5. *Microbial inocula*

Freshly grown bacterial cells were harvested from R2A medium by adding 5 ml of mineral salt medium (MSM: K_2HPO_4 1.4 g/l, KH_2PO_4 1.4 g/l, $MgSO_4{\cdot}7H_2O$ 0.1 g/l, $(NH_4)_2SO_4$ 1.0 g/l, $MnSO_4{\cdot}7H_2O$ 0.001 g/l, and $FeSO_4{\cdot}7H_2O$ 0.01 g/l) to the Petri dishes. The fungal strain *C. ligniaria* F/TGF15 was grown on PDA plates for 12 days at 20 °C. Then, 10 ml of MSM was added per plate and fungal tissue and spores were scraped off with a spatula. The optical density (OD_{600}) of the suspensions was adjusted to 0.1 with MSM using a Beckman DU® 530 Life Science UV/Vis spectrophotometer. Dilutions of 1000-fold were then made to obtain suspensions that contained approximately 10^5 CFU per ml. The densities of the bacterial and fungal inoculants were checked by dilution plating on R2A.

2.6. *Colonization of TGF with single bacteria and bacterial consortia*

The selected bacteria described in Table 1 were tested as single strains for their capacity to colonize TGF in several independent experiments. Survival was determined by plate counts after 3 and 5 days. Bacterial cell densities in TGF were determined by dilution plate counting. For this purpose, 5 ml of 0.85% NaCl solution was added to each TGF microcosm. The treated TGF was shaken for 10 min at maximum speed on a shaker (SF1, Stuart Scientific, Great-Britain). Serial 10-fold dilutions were prepared in sterile saline and spread-plated in triplicate on R2A. Plates were incubated at 25 °C. Colonies were counted every 2 days up to 12 days. The counts were then expressed per g dry TGF and transformed to logarithmic scale.

A bacterial consortium consisting of *r* and *K* strategists was introduced into TGF. We thus combined selected Gram-negative and Gram-positive bacteria, namely *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *S. maltophilia* 34/TGE5, *R. radiobacter* 43/TGE20, *F. denitrificans* 48/TGE20, and *A. aurantiacus* 95/TGF15. Also a consortium containing of six Gram-positive bacteria, namely *Leifsonia poae* 83/TGF15, *L. xyli* subsp. *xyli* 93/TGF15, *Afipia broomeae* 94/TGF15, *L. poae* 97/TGF15, *L. poae* 108/TGF15, and *A. broomeae* 109/TGF15 was prepared by combining these cultures as inocula. Inoculation of TGF was done as described above. Survival was determined by assessing the CFU numbers appearing on plates following dilution plating on the relevant media after 1, 24, and 96 h.

2.7. Colonization of TGF with combinations of bacteria and C. ligniaria F/TGF15

The bacterial strains *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli* subsp. *xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, *A. aurantiacus* 95/TGF15, and a mix of *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, and *M. radiotolerans* 56/TGF10 were combined with *C. ligniaria* F/TGF15 to test their ability to colonize TGF. As a control, *C. ligniaria* F/TGF15 was also introduced to the TGF without inoculant bacteria. Survival of the inoculant bacteria as well as *C. ligniaria* was determined by dilution plate counts on the relevant media after 7 days as described previously.

2.8. Effect of nutrients and surfactant on TGF colonization

To test if the colonization of TGF by bacterial strains was affected by a lack of nutrients or by TGF surface specifics, 1 ml of suspension (containing 10^8 cells per ml) of selected organisms, i.e. (1) *M. radiotolerans* 56/TGF10 or (2) the bacterial consortium with seven strains in roughly equal CFU numbers, was mixed with 0.6 ml of AFY solution (0.5% acetic acid + 0.5% formic acid + 0.001% yeast extract in MSM), 0.6 ml of surfactant (0.01% or 0.001% Silwet in MSM), or a combination of both. These suspensions were added to the TGF. Survival was determined by dilution plate counts on the relevant media (see above) after 7 days.

2.9. Effect of washing and sterilization on TGF colonization

In this experiment, non-sterilized, gamma-irradiated and washed gamma-irradiated TGF samples were used. The non-sterilized TGF was used as a control. The washed gamma-irradiated samples were prepared as follows: 19 g TGF was mixed with 500 ml of distilled water and stirred for 2 h using a magnetic stirrer at 700 rpm. Grass fibers were collected on filter paper and washed once more in distilled water during 15 min. They were dried at room temperature in a flow cabinet for 3 days. These steps were all performed under sterile conditions. A bacterial–fungal consortium consisting of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli* subsp. *xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, *A. aurantiacus* 95/TGF15, and *C. ligniaria* F/TGF15 was prepared by combining roughly equal CFU numbers (about 10^5 per ml final concentration) of all inocula. In some treatments, additional nutrients were added. Thus, 0.8 ml of inoculum was mixed with 0.8 ml of AFY (0.5% acetic acid + 0.5% formic acid + 0.001% yeast extract, in MSM medium) before addition to the TGF. After 7 days incubation, survival was determined by dilution plate counting. Bacterial composition was also assessed by direct molecular means, i.e. by PCR–DGGE performed on DNA directly extracted from TGF.

2.10. Colonization of TGF after pre-colonization by C. ligniaria F/TGF15

TGF was inoculated with 1.6 ml of a *C. ligniaria* F/TGF15 suspension in MSM medium containing approximately 10^4 propagules per ml. The matrix was mixed, and incubated at 25 °C. A bacterial consortium (0.8 ml, containing 10^5 cells per ml) of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli* subsp. *xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, and *A. aurantiacus* 95/TGF15 was introduced either 3 h, or 1, 3, 6, and 13 days later. Seven days after inoculation with the consortium, survival of all inoculants was determined by dilution plate counting on R2A medium. Plates were incubated at 25 °C and checked every 2 days up to 12 days. Colonies of *S. plymuthica* 23/TGE5 and *M. radiotolerans* 56/TGF10 were recognizable by colony morphology and these were thus counted separately. This entire experiment was repeated in two replicates under exactly the same conditions.

Furthermore, the bacterial composition of the TGF was assessed with PCR–DGGE. Additionally, 25 colonies randomly obtained from plates were identified by partial sequencing of the 16S rRNA (Trifonova et al., 2008a).

2.11. Analysis of bacterial community composition in TGF by PCR–DGGE and colony PCR

The bacterial composition of the inoculated TGF was analyzed with PCR–DGGE as follows. DNA was extracted from TGF samples using the UltraClean™ soil DNA kit (MoBio Laboratories, BIOzymTC, Landgraaf, The Netherlands), as described (Trifonova et al., 2008a). The DNA from the four Gram-negative bacteria was isolated as described (Trifonova et al., 2008a) and DNA from the three Gram-positive bacteria was extracted by using the Master Pure Gram Positive DNA Purification Kit (EPICENTRE, USA). DNA of TGF samples and the seven strains was used as templates for PCR amplifying 16S ribosomal (r)RNA genes, followed by DGGE as described (Trifonova et al., 2008a).

PCR products of the 25 single colonies were purified with the High-Pure PCR purification kit (Roche GmbH, Mannheim, Germany) and then sequenced using an ABI Prism automatic sequencer (Greenomics, Wageningen, The Netherlands).

2.12. Statistical analysis

Analyses of variance (ANOVA) were performed on the log-transformed CFU values with the statistical program Genstat 8 (Rothamsted Experimental Station, Harpenden, UK). Least significant differences (LSD) were calculated at a significant level of $P = 0.05$.

3. Results

3.1. In vitro antagonism of selected TGF bacteria towards a range of fungi

To create a TGF substrate which will finally be suppressive against phytopathogenic fungi, *in vitro* antagonism of the 11 selected bacteria towards 10 fungi was tested. From the 11 strains tested, three inhibited one or more fungi (Table 2). Two types of inhibition zones were observed, i.e. (1) a complete inhibition of spore germination or hyphal growth, and (2) a growth reduction resulting in retarded growth. The isolate *S. plymuthica* 23/TGE5 inhibited all 10 fungi tested and *P. corrugata*

Table 2 – *In vitro* antagonism of three bacterial isolates that inhibited one or more fungi.

Isolate no.	Name of closest affiliate	*Fusarium* sp.		*Fusarium oxysporum* sp. *radicis lycopersici*		*Fusarium oxysporum* sp. *radicis lycopersici*		*Penicillium* sp.		*Pythium ultimum*		*Pythium aphani dermatum*		*Rhizoctonia solani*		*Rhizopus* sp.		*Trichoderma harzianum*		*Verticillium dahliae*	
		1[a]	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
23/TGE5	*S. plymuthica*	+[b]	–	–	+	–	++	++	+++	+	–	+	–	+++	–	+	–	+	++	+	++
31/TGE5	*P. corrugata*	–	+	–	+	–	+++	–	–	++	–	–	+	+	+++	+	–	–	+	+++	++
34/TGE5	*S. maltophilia*	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–

[a] 1, complete inhibition of fungal spore germination or mycelium growth; 2, partial inhibition, slower growth and germination.

[b] Size of inhibition zones: +++, >10 mm; ++, 5–10 mm; +, 0–4 mm; –, no inhibition zone.

31/TGE5 inhibited 9 of the fungi tested (except *Penicillium* sp.), whereas isolate *S. maltophilia* 34/TGE5 only partially inhibited 2 in 10 tested fungi (Table 2). The other bacterial isolates did not inhibit any of the tested fungi.

3.2. *Colonization of TGF with single bacteria and bacterial consortia*

Following introduction of approximately 10^5 CFU per g matrix, none of the 11 selected bacterial strains survived after 3 or 5 days in TGF, as in all cases their CFU numbers dropped to below detection (estimated at 200 CFU per g). Also, the consortium of the six selected Gram-positive bacteria did not survive after 3 and 5 days presence in the matrix (data not shown).

The consortium of the seven selected bacterial strains *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli* subsp. *xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, and *A. aurantiacus* 95/TGF15 also did not show any persistence as culturable forms in the matrix (3 and 5 days, detection limit 200 CFU per g). A very similar result was found when combined Gram-negative and Gram-positive bacteria were used, in the consortium consisting of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *S. maltophilia* 34/TGE5, *R. radiobacter* 43/TGE20, *F. denitrificans* 48/TGE20, and *A. aurantiacus* 95/TGF15. In this case, measurements taken shortly following the introduction 1 and 24 h revealed that the population as a whole gradually decreased after its introduction at 8×10^6 CFU per g into the TGF. After 3 days the population was below log 2 CFU, after 96 h no CFU were detectable anymore. The progressive CFU death was consistent across several experiments. Overall, these data show that the TGF matrix exhibits a generally bactericidal effect on bacterial inoculants.

3.3. *Colonization of TGF with combinations of bacterial strains and C. ligniaria F/TGF15*

Table 3 summarizes the bacterial and fungal numbers present in the TGF after 7 days of incubation. Three days after introduction, each of the bacterial strains tested was already below the limit of detection, i.e. log 2 CFU per g dry TGF. Seven days after introduction, in the combination with *C. ligniaria*, none of the bacterial strains was detectable anymore.

In contrast, *C. ligniaria* F/TGF15 revealed growth in the TGF matrix, from approximately log 4.5 per g dry material at the onset of the experiment to over log 9.4 CFU per g dry TGF after 7 days. Similar fungal growth was found in all cases, i.e. upon single introduction or in the combination with any of the bacterial strains.

3.4. *Colonization of TGF by bacterial strains and C. ligniaria F/TGF15 under varied conditions*

Table 4 summarizes the data on the colonization of differently treated TGF samples. Again, *C. ligniaria* F/TGF15 was able to grow on TGF in all treatments, from log 4.7 CFU per g up to log 8.6–log 9.9 CFU per g dry TGF. As expected, all bacterial inoculants died out as CFU within 3 days. Prior gamma sterilization of the TGF did not exert any effect on the bacterial

Table 3 – Colonization of TGF after inoculation with the fungus *Coniochaeta ligniaria* F/TGF15 alone, or in combination with single bacterial isolates or a bacterial mix. Populations are measured after 7 days incubation at 25 °C.

Inoculated isolates	Bacterial inoculum (log CFU per g dry TGF)	Bacterial population (log CFU per g dry TGF)	Fungal inoculum (log CFU per g dry TGF)	Fungal population (log CFU per g dry TGF)
F/TGF15	–	–	4.3	10.5
15/TGE5 + F/TGF15	5.6	<2[a]	4.8	10.1
23/TGE5 + F/TGF15	5.6	<2[a]	4.8	11.2
31/TGE5 + F/TGF15	5.6	<2[a]	4.3	10.3
56/TGF10 + F/TGF15	5.6	<2[a]	4.8	9.4
66/TGF15 + F/TGF15	5.6	<2[a]	4.8	9.7
70/TGF15 + F/TGF15	5.6	<2[a]	4.3	11.7
95/TGF15 + F/TGF15	5.6	<2[a]	4.3	12.2
23/TGE5 + 31/TGE5 + 56/TGF10 + F/TGF15	5.6	<2[a]	4.8	10.2
LSD[b]				0.4

[a] Detection limit of the method is log 2 CFU per g TGF.

[b] Least significant difference at $P = 0.05$.

colonization, irrespective of the presence of *C. ligniaria* F/TGF15. However, washing of TGF did enhanced the colonization of TGF with all bacterial inoculants tested: following inoculation with approximately log 5 CFU per g, at the end of the experiment (7 days) log 8.4 CFU were present in sterilized washed TGF, whereas log 7.0 CFU appeared in sterilized washed TGF with *C. ligniaria* F/TGF15 (Table 4). The addition of extra (0.5% acetic acid + 0.5% formic acid + 0.001% yeast extract in MSM) nutrients or the combined application with the fungus did not enhance colonization since the final number of persisting bacteria was reduced (only log 6.4 CFU bacteria).

Direct analysis of the bacterial community structure in TGF with PCR–DGGE of matrix-extracted DNA (Fig. 1) revealed that almost all of the seven introduced strains in the washed, washed-plus-fungus and washed-plus-fungus-plus-nutrients treatments could be tracked back. Strains *S. plymuthica* 23/TGE5 (band 1), *P. putida* 15/TGE5, or *P. corrugata* 31/TGE5 (band 6) appeared to be relatively dominant. The only exception was *M. radiotolerans* 56/TGF10, of which the presence could not be proven, since band 7 corresponded also to isolate *P. corrugata* 31/TGE5.

3.5. *Colonization of TGF by a bacterial consortium after pre-colonization with C. ligniaria F/TGF15*

Since *C. ligniaria* F/TGF15 was consistently able to colonize TGF, and in the light of its proven ability to break down phytotoxic compounds (Trifonova et al., 2008b), TGF was pre-colonized with this fungus for different periods of time, on the assumption that this pre-growth might also remove the putative bactericidal compounds from the matrix. Thereafter, the consortium of seven selected bacterial strains was added at log 5.8 CFU per g dry TGF. The results showed that after 3, 6, and 13 days of pre-colonization with the fungus, the consortium could grow in the TGF, from about log 5.8 up to about log 10 CFU per g TGF (Table 5). Approximately 10% of this population consisted of *S. plymuthica* 23/TGE5 and/or *M. radiotolerans* 56/TGF10, as evidenced from the pink colonies appearing on plates (Table 5). On the other hand, pre-colonization of the TGF by *C. ligniaria* for just 3 h or 1 day did not facilitate bacterial growth or persistence, as the CFU counts dropped to below the detection limit, i.e. log 2 cells per g dry TGF.

The 25 random colonies that were checked for their identity by colony PCR, were *P. putida* 15/TGE5 12× (48%) of the

Table 4 – Colonization of differently treated TGF after inoculation with the fungus (F) *Coniochaeta ligniaria* F/TGF15 in combination with a bacterial mix (B mix) of isolates *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli* subsp. *xyli* 66/TGF15, *M. anthracenicum* 70/TGF15, and *A. aurantiacus* 95/TGF15. Populations are measured after 7 days incubation at 25 °C.

Treatments of TGF			Microbial inoculum	Bacterial inoculum (log CFU per g dry TGF)	Bacterial population (log CFU per g dry TGF)	Fungal inoculum (log CFU per g dry TGF)	Fungal population (log CFU per g dry TGF)
–	–	–	B mix + F	5.3	<2[a]	4.7	8.9
Sterilized	–	–	B mix + F	5.3	<2[a]	4.7	8.6
Sterilized	Washed	–	B mix	5.3	8.4	ND[b]	ND[b]
Sterilized	Washed	–	B mix + F	5.3	7.0	4.7	9.2
Sterilized	Washed	Nutrients	B mix + F	5.3	6.4	4.7	9.9
LSD[c]					0.3		1.0

[a] Detection limit of the method is log 2 CFU per g TGF.

[b] Not determined.

[c] Least significant difference at $P = 0.05$.

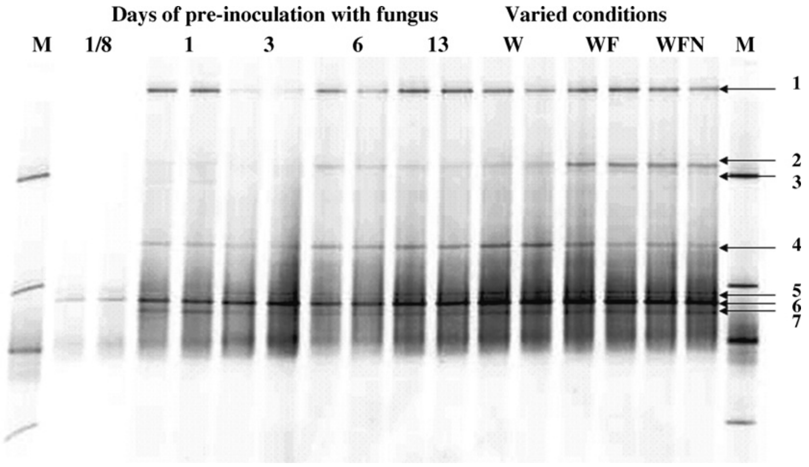

Fig. 1 – Fingerprint by PCR–DGGE of the bacterial communities in differently treated TGF samples. TGF was pre-inoculated with the fungus *Coniochaeta ligniaria* F/TGF15 1/8, 1, 3, 6, and 13 days before the bacterial mix was added. TGF samples were washed (W), with or without added nutrients (N) and inoculated together with the fungus (F). The bacterial mix consisted of 15/TGE5, 23/TGE5, 31/TGE5, 56/TGF10, 66/TGF10, 70/TGF15, and 95/TGF15. "M" is a general bacterial marker. The band numbers corresponded with the following strains: 1, strain 23/TGE5; 2, strain 66/TGF10; 3, strain 70/TGF15; 4, strain 95/TGF15; 5, strain 15/TGE5; 6, strains 15/TGE5 and 31/TGE5; 7, strains 31/TGE5 and 56/TGF10.

population, *S. plymuthica* 23TGE5 3× (12%), *P. corrugata* 31/TGE5 1 × (4%), and *M. radiotolerans* 56/TGF10 5× (20%). Hence, at least four species were found to persist, as dominating members of the community that yielded CFU. DNA extraction or PCR from four colonies failed, and these might be the Gram-positive strains *L. xyli* subsp. *xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15. The latter were still present in the community in the TGF matrix, as they were found by PCR–DGGE analysis as bands 2 and 4 (Fig. 1).

4. Discussion

Eleven bacterial strains and one fungus, initially isolated from enrichments in TGF or its extract, were selected to actively colonize freshly prepared TGF, in an attempt to establish a plant-beneficial microbial community on this substrate. As several bacterial strains originated from sequential enrichment cultures in the TGF itself (Trifonova et al., 2008a), it was expected that these strains would easily colonize the TGF.

Table 5 – Colonization of TGF after pre-inoculation with the fungus *Coniochaeta ligniaria* F/TGF15 during 1/8, 1, 3, 6, and 13 days by the bacterial mix of isolates 15/TGE5, 23/TGE5, 31/TGE5, 56/TGF10, 66/TGF15, 70/TGF15, and 95/TGF15. Populations are measured after 7 days incubation at 25 °C in two independent experiments.

Pre-colonization time with fungus (days)	Bacterial inoculum (log CFU/g dry TGF)	Bacterial population (log CFU per g dry TGF)	Pink bacterial colonies[b] (log CFU per g dry TGF)	Fungal inoculum (log CFU per g dry TGF)	Fungal population (log CFU per g dry TGF)
Experiment 1					
1/8	5.7	<2[a]	2[a]	4.6	9.1
1	5.7	<2[a]	2[a]	4.6	10.8
3	5.7	10.5	8.8	4.5	10.1
6	5.7	10.3	9.3	4.3	10.5
13	5.7	10.4	8.6	4.4	10.5
LSD[c]		0.3			0.6
Experiment 2					
1/8	5.9	<2[a]	<2[a]	3.9	9.1
1	5.9	<2[a]	<2[a]	3.9	8.9
3	5.9	10.0	9.0	4.7	8.9
6	5.9	9.8	8.9	4.1	9.2
13	5.9	9.8	7.5	4.4	9.1
LSD[c]		0.4			0.6

[a] Detection limit of the method is log 2 CFU/g TGF.

[b] Isolates 23/TGE5 and 56/TGF10 both form pink colonies.

[c] Least significant difference at $P = 0.05$.

Unexpectedly though, TGF was found to be very recalcitrant to bacterial colonization. None of the selected bacteria, applied either as single strains or as combinations of strains (consortia), was able to colonize and persist in the matrix. This analysis included various Gram-negative and Gram-positive bacteria, the latter including actinobacteria which abounded in the enrichment cultures of TGF (Trifonova et al., 2008a). In fact, these bacteria all showed rapid declines of CFUs following their introduction into TGF.

We first hypothesized there might have been a necessity for easily available nutrients in the colonization process or, alternatively, a problem with a too hydrophobic nature of the fibers. However, addition of selected nutrients or of a surfactant did not enhance the survival of any of the bacterial inoculants in the TGF. We then surmised that TGF might contain bacteriostatic or bactericidal factors of unknown nature that could possibly be removed by cold water washing. Indeed, washing of the TGF allowed several bacterial inoculants, namely *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, and *M. radiotolerans* 56/TGF10 to establish and grow on the TGF. Hence, we concluded that TGF contained, as-yet-unknown water-soluble compounds that are detrimental to the persistence and growth of the selected bacteria.

In contrast to the bacterial strains, the fungus *C. ligniaria* was shown to consistently grow and persist on TGF. In the previous enrichment experiments, this fungus had been detected with fungal PCR–DGGE in the subsequent steps of TGF and its extracts. It thus turned out to be a key element in our further developmental research. Previous research had already shown the capacity of *C. ligniaria* to break down several phytotoxic compounds that are present in TGF extract, including phenol, 2,6-methoxyphenol and 2-furalaldehyde (Trifonova et al., 2008b). *C. ligniaria* is also known to play a role in the biological detoxification of lignocellulosic hydrolysates (López et al., 2004, 2007). In natural environments, *C. ligniaria* has been found on pine sapwood samples (Raberg et al., 2007). It has also been studied in bioabatement processes as a potential agent for removing inhibitory compounds from lignocellulose hydrolysates (Nichols et al., 2005).

Pre-colonization of TGF for 3 days or more with *C. ligniaria* was shown to pave the way for subsequent bacterial growth on TGF. The most likely explanation for this effect is that the concentrations of key compounds that inhibited bacterial colonization and growth were strongly reduced by the fungal growth. This hallmark finding provides an indication for the role of *C. ligniaria* as a mediator organism, promoting bacterial growth by removing bacteriostatic or bactericidal compounds from TGF. Thus, *C. ligniaria* created appropriate growth conditions for bacteria. Belessi et al. (2008) also observed bacterial growth influenced by fungi; pH increase due to fungal growth enhanced survival of *Listeria innocula*. In soil, filamentous fungi can favor the distribution of bacteria by providing continuous surfaces and channels on or within which bacteria can move (Kohlmeier et al., 2005). The strategy of combining fungal and bacterial inoculants to achieve both establishment and persistence of inoculants can have important repercussions in the applied areas of ecology, e.g. in bioremediation.

The cultural bacterial community that persisted in the *C. ligniaria*-treated TGF consisted of minimally four strains, i.e. *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, and *M. radiotolerans* 56/TGF10. All are Gram-negative bacteria, three of them being *r*-strategists and one, *M. radiotolerans* 56/TGF10, a *K*-strategist. Moreover, strains *L. xyli* subsp. *xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, and *A. aurantiacus* 95/TGF15 which are all Gram-positive bacteria, although not found in the 25 random isolates, were definitely present in the community, as their bands were found in the DGGE banding pattern.

The four Gram-negative bacterial strains which persisted in the TGF all have plant-health-promoting potential. Strain 23/TGE5, identified as a *S. plymuthica*, had *in vitro* antagonism towards several phytopathogenic fungi, i.e. *R. solani*, *F. oxysporum* sp. *radicis lycopersici*, *V. dahliae*, *P. aphanidermatum*, and *P. ultimum*. Species of *Serratia* encompass many biocontrol agents (Jeun et al., 2007; Ovadis et al., 2004; Roberts et al., 2007; Someya et al., 2007) and they are prime producers of the antibiotic pyrrolnitrin (Garbeva et al., 2004b; Liu et al., 2007; Meziane et al., 2006). Also strain 31/TGE5, identified as *P. corrugata*, showed *in vitro* antagonism towards all aforementioned phytopathogenic fungi. *Pseudomonas* strains with antagonistic properties against soil-borne pathogens have been studied intensively (Deacon and Berry, 1993; Garbeva et al., 2004a,b). *P. corrugata* strains are known as antagonists of *Botrytis cinerea* (Guo et al., 2007), *P. aphanidermatum*, and *P. ultimum* (Georgakopoulos et al., 2002; Gravel et al., 2005; Schmidt et al., 2004), *Ralstonia solanacearum biovar* 2 (Van Overbeek et al., 2002), *F. oxysporum* (Elmer, 1995; Schisler et al., 1997), and *Monilinia fructicola* (Smilanick et al., 1993). Although no fungal plant pathogen inhibition was measured with the isolates *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10, those bacteria are potential plant-beneficial bacteria (Guo et al., 2007; Zarnowski et al., 2002; Kai et al., 2007).

5. Conclusion

We aimed to valorize the plant growth substrate TGF with a stable microbial consortium consisting of *r* and *K* strategists and with the potential to improve plant health. The initial colonization of TGF by *C. ligniaria* F/TGF15 was found to be crucial to allow establishment of bacterial beneficials. A consortium of seven bacterial strains, four of which well known as plant-health-promoting species, was thus consistently established, at high CFU numbers, in the TGF matrix. This indicates the potential of microbiologically matured TGF as a new substrate allowing the stable and consistent introduction of a vital plant-health-improving microbial consortium. We are currently testing the effect of the presence of the microbial consortium in the TGF matrix on the growth and health of plants supported by it.

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